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Original Article

Macrophage migration inhibitory factor promotes osteosarcoma growth and lung metastasis through activating the RAS/MAPK pathway

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ABSTRACT

Emerging evidence suggests that the tumour microenvironment plays a critical role in osteosarcoma (OS) development. Thus, cytokine immunotherapy could be a novel strategy for OS treatment. In this study, we explored the role of macrophage migration inhibitory factor (MIF), an important cytokine in OS progression, and investigated the anti-tumour effects of targeting MIF in OS. The results showed that MIF significantly increased in the tissue and serum samples of OS patients and was associated with tumour size, pulmonary metastasis and the survival rate of OS patients. We verified a positive correlation between MIF and p-ERK1/2 in OS patients. The *in vitro* results indicated that MIF could activate the RAS/MAPK pathway in a time- and dose-dependent manner, thereby promoting cell proliferation and migration. Furthermore, shRNA targeting MIF significantly inhibited tumour growth and lung metastasis in a mouse xenograft model and orthotopic model of OS. Additionally, inhibition of MIF significantly enhanced the sensitivity of OS cells to cisplatin and doxorubicin. Our findings suggest that immunotherapy targeting MIF to block the RAS/MAPK kinase cascade may represent a feasible and promising approach for OS treatment.

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Introduction

Osteosarcoma (OS) is the most common primary bone cancer and remains a leading cause of cancer-related deaths in adolescents [1,2]. Although a combination treatment based on surgical resection and aggressive adjuvant chemotherapy has been adopted for the treatment of OS over the past decades, disease relapse and pulmonary metastasis are still challenges in OS clinical therapy [3]. Thus, developing more effective therapeutic approaches for OS clinical trials is urgently needed.

Increasing evidence has suggested that the tumour microenvironment plays a critical role in driving cancer development; thus, cancer immunotherapy, such as cytokine therapy, could be a novel strategy for OS treatment [4,5]. As a pleiotropic cytokine, macrophage migration inhibitory factor (MIF) is constitutively expressed by immune and endocrine cells and also by epithelial cells, which are in direct contact with the external environment [6]. Recent studies have shown that MIF is an important effector of the innate immune system and also contributes to tumour progression and malignant transformation [7,8]. MIF has been reported to be involved in breast cancer development, and MIF over-expression could promote breast cancer metastasis by regulating the HMGB1/TLR4/NF kappa B axis [9,10]. Increased expression of MIF was found in human and $Apc^{Min/+}$mouse intestinal adenomas and could promote intestinal tumourigenesis and metastasis [11,12]. An

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oncogenic role of MIF was also identified in tumour cells of non-small cell lung cancer [13], hepatocellular carcinoma [14], melanoma [15] and bladder cancer [16]. Han et al. found that the up-regulated MIF was correlated with worse prognosis in high-grade human OSs [17]. Nevertheless, the biological role of MIF in OS remains unclear, and MIF has not been used as a potential direct target to successfully treat OS.

In the present study, we investigated the correlation between MIF levels and the clinicopathological parameters of 60 OS patients. Increased expression of MIF in tissue and serum samples of OS patients was further verified. The effect of MIF on the RAS/MAPK pathway, cellular proliferation and migration was revealed in human OS 143B cells and MNNG/HOS cells. Furthermore, the anti-tumour effects of MIF knockdown were studied in mouse xenograft and orthotopic models of OS based on lentivirus-mediated short-hairpin RNA interference.

Materials and methods

Clinical samples and cell lines

Clinical samples were obtained from 60 OS patients who underwent radical resection at Jinling Hospital (Nanjing, P. R. China) from 2008 to 2012. Serum samples were obtained from a subset of 35 patients at the time of diagnosis. Thirty-five disease-free volunteers were selected as the healthy controls. A protocol concerning the use of patient samples in this study was approved by the Medical Ethics Committee of the affiliated Jinling hospital of Nanjing University (Registration No. 2008NLY-045). Signed informed consent was obtained from each patient. OS patient clinical information is listed in Table 1. A follow-up survey of the OS patients was performed for three years after the diagnosis.

MNNG/HOS cells and 143B cells were cultured in MEM medium with low glucose containing 10% FBS (Thermo Scientific, Grand Island, NY, USA). All cell lines were obtained from the Institute of Cell Biology at the Chinese Academy of Sciences (Shanghai, China).

Histology and immunohistochemical (IHC) analyses

As previously described [18], the tissues were fixed in 4% (wt/vol) paraformaldehyde. After the samples were paraffin-embedded, 5-μm sections were cut and stained with haematoxylin and eosin (H&E) (Sigma-Aldrich, St. Louis, MO, USA). The deparaffinized 5-μm sections were incubated with rabbit polyclonal anti-MIF antibody (Santa Cruz, dilution ratio: 1:100) at 4 °C overnight after antigen retrieval. The sections were then stained by the avidin-biotin-peroxidase complex using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The peroxidase reaction was visualized by incubating the sections with 3-amino-9-ethylcarbazole solution, and counterstaining was carried out using Mayer's haematoxylin (Sigma-Aldrich).

Cell treatments

Cells (2×10^5) were seeded in 6-well plates overnight. Then, cells were cultured in serum-free MEM for 12 h and treated with rMIF (PeproTech) at various concentrations (0, 50, 100 ng/mL) for different times (0 min, 10 min, 30 min, 2 h, 6 h). Additionally, cells were treated with 100 ng/mL rMIF for 2 h in the presence or absence of 10 mM U0126 (MEK1/2 inhibitor, Sigma-Aldrich) for the following protein and RNA analysis.

Construction of stable cell lines

Cells were infected with recombinant lentiviruses in the presence of 8 μg/mL polybrene for 12 h. Then, the medium was replaced by the fresh medium containing 10 μg/mL puromycin. Finally, the stable cell lines of shRNA-MIF-LV-143B and shRNA-MIF-LV-MNNG/HOS and their control cells (shRNA-NC-LV-143B and shRNA-NC-LV-MNNG/HOS) were established. Four sequences of shRNAs (Sangon Biotech) for MIF are listed in Supplementary Table 3. The most effective sequence is TGCTGACCACGTGCACCGCGATGTACGTTTTGGCCACTGACTGACGTACATCGGTGCACGTGGT.

Western blot analysis and qRT-PCR assay

Western blot analysis was carried out as previously described [18]. The antibodies used in this study are listed in Supplementary Table 1. Total RNA was prepared using TRIzol reagent (Life Technologies). The qRT-PCR assay was performed using a One Step SYBR PrimeScript RT-PCR Kit (TaKaRa, Shiga, Japan). β-actin was used as an internal control for gene expression. All primers for qRT-PCR are listed in Supplementary Table 2.

Establishment of a mouse OS xenograft model

A mouse OS xenograft model was established in four-week-old thymic BALB/c male nude mice purchased from the Laboratory Animal Centre of Nanjing University (Nanjing, China). All animals received care according to the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health. Briefly, 1×10^7 stable cells in 100 μl PBS were injected subcutaneously into the right flanks of mice. The tumours were measured on days 9, 12, 15, 18, 21, 24, 27 and 30. The tumour volume was calculated as $\frac{1}{2}LW^2$, where W and L are the smallest and the largest perpendicular tumour diameter, respectively. The mice were sacrificed and photographed at 30 days post-implantation.

Establishment of an orthotopic OS model

An orthotopic model of OS was constructed in four-week-old thymic BALB/c male nude mice as described by Luu et al. [19]. The 143B stable cells (1×10^6 cells in 20 μl PBS) were injected percutaneously into the tibia of anesthetized nude mice. For

Table 1
Demographics and clinical variables.

Clinical variables	MIF level in tissue				MIF level in serum		
	n	Low	High	P (χ^2 test)	n	MIF (ng/mL)	P (χ^2 test)
Sex				0.282			0.532
Male	40	16	24		23	1.520 ± 0.431	
Female	20	6	14		12	1.527 ± 0.546	
Age				0.063			0.637
≤17	34	16	18		19	1.530 ± 0.402	
>17	26	6	20		16	1.514 ± 0.605	
Location				0.687			0.874
Femur	33	14	19		17	1.515 ± 0.416	
Tibia	24	7	17		10	1.464 ± 0.537	
Other	3	1	2		8	1.532 ± 0.472	
Histologic subtype				0.215			0.312
Osteoblastic	36	12	24		19	1.441 ± 0.403	
Chondroblastic	13	5	8		8	1.701 ± 0.522	
Fibroblastic	5	2	3		3	1.547 ± 0.242	
Telangiectatic	4	2	2		3	1.647 ± 0.344	
Other	2	1	1		2	1.428 ± 0.204	
Enneking stage				0.271			0.014
IIB	58	22	36		33	1.468 ± 0.517	
III	2	0	2		2	2.549 ± 0.103	
Tumour size (cm)				0.009			0.003
≥5	39	13	26		22	1.726 ± 0.413	
<5	21	9	12		13	1.217 ± 0.406	
Pulmonary metastasis				0.008			0.001
Yes	18	2	16		16	2.042 ± 0.234	
No	42	20	22		19	1.107 ± 0.354	

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